SUPPLEMENTAL DATA

Insights in ChAdOx1 nCov-19 Vaccine-induced Immune Thrombotic Thrombocytopenia (VITT)

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SUPPLEMENTAL MATERIALS AND METHODS

Materials

100 µg of mouse monoclonal IgG2a antibody raised against the adenovirus hexon polypeptide (Abcam, ab7428, B025/AD51) was Cy5-conjugated using the Lightning-Link Cy5 conjugation kit, according to manufacturer's instructions (Novus Biologicals, USA). For staining of PF4, mouse monoclonal IgG2b (RTO clone, Thermo Fisher, USA; MA5-17639) antibody was used. PF4-affinity-purified IgG from VITT sera was primarily Cy5-conjugated using the Lightning-Link kit. For immobilization of viral particles and PF4, 1.5 high-precision coverslips (VWR, Germany) were cleaned by sonication for 1 h in 96% ethanol, 1 h in 1M HCl, 1 h in 1M NaOH and 1 h in distilled water. ChAdOx1 nCoV-19 vaccine was diluted 1:10 in 0.9% NaCl and 0.2% sucrose in H_2O and human PF4 was added to a final concentration of 1 µg/mL. Five µL of the mixture were spread on a cleaned coverslip and air-dried. Fixation and immunofluorescence staining were performed as described below but with 0.5 µg/mL PF4 (RTO clone) antibody and 30 min antibody incubation.

Platelet rich plasma (PRP) from healthy donors was incubated with 10 µg/mL PF4, 1:10 ChAdOx1 nCoV-19 vaccine diluted in 0.9% NaCl, and 0.2% sucrose in distilled water for 15 min at 37°C. As negative control, PRP diluted in saline/sucrose was used. Platelets were allowed to adhere on coverslips for 15 min at 37°C, and were subsequently fixed for 10 min with 2% EM-grade paraformaldehyde in 80 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, 4% sucrose, pH 6.8 at RT. After one wash in 1x PBS, slides were blocked with 2% fetal bovine serum, 2% bovine serum albumin, 0.1% cold fish gelatin and 2% normal goat serum in 1x PBS pH 7.4, for 45 min at RT. Primary anti-PF4 antibody (10 µg/mL) was added to the blocking

solution and incubated with platelets for 1 h at RT. After 4 washes with 1x PBS, bound antibodies were detected by goat anti-mouse AlexaFluor 488-conjugated secondary antibody (1:500 dilution; Thermo Fisher Scientific) for 45 min at RT. Following several washing steps, 1 µg/mL monoclonal Cy5-conjugated anti-adenovirus antibody was applied for 1 h at RT. In respective experiments, Cy5-conjugated VITT-IgG was diluted to 10 µg/mL and applied to the immobilized PF4-adenoviral complexes for 1 h at room temperature. Cells were washed and kept moist in 1x PBS, and slides were stored in the dark at 4°C until imaging.

Super-Resolution Localization Microscopy (dSTORM)

Before mounting, samples were washed once in distilled water. Next, 100 nm Tetraspek fluorescent beads (Thermo Fisher Scientific), diluted 1:800 in ultrapure water, were added for 10 min to each sample as fiducial markers. After one wash in ultrapure water, samples were mounted inversely in Everspark dSTORM buffer (Idylle Labs, Paris, France) on depression glass slides. Coverslips were rapidly sealed with TwinSil two component silicon (Picodent GmbH, Wipperfürth, Germany). To avoid overt oxygenation, care was taken to ensure that mounting times were faster than 30 s. Sealed slides were imaged on the above-described Zeiss Elyra PS.1 system. Sequentially, 10,000 to 14,000 frames with a size of 200x200 pixels were acquired in each channel using TIRF-HP illumination with 100% laser intensity of the 647 and 488 nm laser lines, respectively. An integration time of 12 ms was used with the definite focus system to compensate z-drift. Raw image stacks were imported to FIJI and fluorescent blinking was detected by Thunderstorm, [1] using Phasor-based 2D localization with a fitting radius of 3 pixels. Localization data were exported as .csv files, density-filtered with a 50 nm radius and x-y-drift corrected, using the fiducial markers listed above. Data was analyzed using customized FIJI [2] and NIS scripts.

Zeta potential measurements

Surface zeta potential (ζ, mV) was performed in folded capillary zeta cell (DTS1070, Malvern Instruments Ltd., Malvern, UK) and consisted of three runs, each with 20 measurements at a voltage set to 10 V. All measurements were performed at the dispersant conductivity of 1.77 ± 0.07 mS/cm (n=45 individual zeta potential runs). Data analysis was performed using Zetasizer software, Version 7.13 (Malvern Instruments Ltd., Malvern, UK). Statistical analysis and data plots were prepared with GraphPad Prism version 9.0.0 for Windows. Differences between groups were considered significant after assessment by ordinary one-way ANOVA with Sidak's multiple comparisons test.

1D-SDS PAGE analysis

For protein separation, 10 µL of 4x SDS-PAGE sample buffer were added to 30 µL of each ChAdOx1 nCoV-19 vaccine lot. Samples and standard (PageRuler Prestained Protein Ladder, Invitrogen/Thermo Fisher) were denatured for 5 min at 95°C and loaded to a precast SDS gel (NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Invitrogen/Thermo Fisher). Electrophoresis was performed at 150 V using a Power Pack 200 (BioRad, Hercules, CA, USA) and a Criterion Cell (BioRad). All reagents and buffers were used according to the manufacturer's instructions. To visualize proteins, silver nitrate staining was performed, as previously described by Shevchenko et al.[3] Gel images were digitalized using a digital camera.

Proteomics and immunoproteomics

SDS was added to vaccine samples to a final concentration of 1% (w/v) to solubilize microparticles. For determination of protein concentrations, a micro-BCA assay was used according to the manufacturer's recommendations (Pierce Thermo Fisher, Bonn, Germany). Next, 5 µg of protein were reduced (2.5 mM DTT ultrapure, Invitrogen/Thermo Fisher, Bonn, Germany) for 30 minutes at 37°C and alkylated (10 mM iodoacetamide for 15 minutes at 37°C, Sigma Aldrich, Munich, Germany). Subsequently, protein was precipitated and digested with trypsin (enzyme to protein ratio of 1:25) on SP3 beads, as described by Blankenburg et al.[4] Lyophilized PF4 was reconstituted in distilled water (4 µg/µL) and 4 µg of protein was diluted in 20 mM ammonium bicarbonate containing 5% SDS, to ensure re-solubilization of protein aggregates. Tryptic peptide lysates were prepared using the SP3-based protocol described above.

An Exploris 480 mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Ultimate 3000 nano HPLC (Dionex/ Thermo Scientific, Waltham, MA, USA) was used for LC-MS/MS experiments. Chromatographic separation of tryptic peptides was achieved by a 60 min linear gradient (30 min for PF4 analysis) using a binary buffer system that consisted of: 0.1% acetic acid in HPLC-grade water; 100% ACN in 0.1% (v/v) acetic acid with increasing concentrations of acetonitrile (7-25% (v/v) in 0.1% (w/v) acetic acid) on a reverse phase column (Accucore 150-C18, 25 cm x 75 µm, 2.6 µm C18, 150 Å), at a flow rate of 300 nL/min at 40°C. The MS scans were carried out in a m/z range of 350 to 1200 m/z. For data acquisition in data independent mode (DIA) (ChAdOx1 nCoV-19 vaccine LOT analysis), precursor scans were acquired at a resolution of 120,000 and fragments at a resolution of 30.000 in 66 windows with 13 m/z and a window overlap of 2 m/z. Data dependent acquisition (DDA; PF4 analysis)

was carried out at a resolution of 120,000 and a normalized AGC target of 3E6 for MS scans. MS/MS spectra were recorded for the top 20 most abundant precursors at a resolution of 15.000 in isolation windows of 1.4 m/z and standard ACG target. Repeated fragmentation of precursor ions was avoided by setting the dynamic exclusion to 10 seconds, HCD collision energy was set to 30 eV.

Protein identification and quantification of data acquired in DDA mode was performed in Proteome Discoverer software (version 2.4, Thermo Scientific) using the SequestHT algorithm. The search was conducted against a combined database limited to human entries (Uniprot/Swissprot version 01/2021) and a processed PF4 protein amino acid sequence (32-101aa) with the following search parameters applied: 10 ppm of mass tolerance for precursor ions and 0.02 Da for fragment-ions; specified enzyme: trypsin; missed cleavages: 2; fixed modification: carbamidomethylation at cysteine; variable modification: methionine oxidation. Only high-confidence peptides (FDR <1%) based on the target-decoy approach were considered for protein group definition. Protein intensities were derived from extracted precursor intensities at a S/N value 1.5.

Data acquired in DIA mode were analyzed with Spectronaut version 14.10.201222.47784 (Biognosis, Zurich, Switzerland) in direct DIA mode using the human Uniprot database (version 01/2021) with added SARS Cov2 spike protein (YP_009724390. 1) sequence and protein sequences of ChAdOx1 nCoV-19 vector [5] consisting of chimpanzee adenovirus Y25 [6] (NC_017825) with exchanged regions (E4ORF4, E4ORF6, E4ORF6/7) from human adenovirus 5 (AC_000008). Identifications were based on a precursor Q-value cut-off of 0.001 and a $FDR_{protein}$ of 0.01. The complete Spectronaut parameters are listed in **Supplemental Table S2**.

Supplemental Table S2: Spectronaut parameters.

Parameter level	Parameter	Setting
Peptides	Toggle N-terminal M	TRUE
Peptides	Min Peptide Length	7
Peptides	Max Peptide Length	52
Peptides	Missed Cleavages	2
Peptides	Digest Type	Specific
Peptides	Enzymes / Cleavage Rules	Trypsin/P
Data Extraction	MS1 Mass Tolerance Strategy	Dynamic
Data Extraction	MS1 Mass Tolerance Strategy - Correction Factor	1
Data Extraction	MS2 Mass Tolerance Strategy	Dynamic
Data Extraction	MS2 Mass Tolerance Strategy - Correction Factor	1
XIC Extraction	XIC IM Extraction Window	Dynamic
XIC Extraction	XIC IM Extraction Window - Correction Factor	1
XIC Extraction	RT IM Extraction Window	Dynamic
XIC Extraction	RT IM Extraction Window - Correction Factor	1

Modifications	Max Variable Modifications	5
Modifications	Select Modifications - fixed Modifications	Carbamidomethyl (C)
Modifications	Select Modifications - variable Modifications	Oxidation (M)
Calibration	MS1 Mass Tolerance Strategy	System Default
Calibration	MS2 Mass Tolerance Strategy	System Default
Identification	Machine Learning	Per Run
Identification	Precursor PEP Cutoff	1
Identification	Protein Qvalue Cutoff	0.01
Identification	Exclude Single Hit Proteins	FALSE
Identification	PTM Localization	TRUE
Identification	PTM Localization - Probability Cutoff	0.75
Identification	Pvalue Estimator	Kernel Density Estimator
Identification	Precursor Qvalue Cutoff	0.001
Identification	Single Hit Definition	By Stripped Sequence
Quantification	Interference Correction	TRUE
Quantification	Best N Fragments per Peptide	TRUE
Quantification	Best N Fragments per Peptide - Min	6
Quantification	Best N Fragments per Peptide - Max	10
Quantification	Quantity MS-Level	MS2
Quantification	Quantity Type	Area
Quantification	Data Filtering	Qvalue sparse
Quantification	Data Filtering - Imputing Strategy	No Imputing
Quantification	Cross Run Normalization	FALSE
Workflow	MS2 DeMultiplexing	Automatic
Workflow	Run Limit for directDIA Library	-1
Workflow	Profiling Strategy	iRT Profiling
Workflow	Profiling Strategy - Profiling Row Selection	Minimum Qvalue Row Selection
Workflow	Profiling Strategy - Profiling Row Selection - Qvalue Threshold	0.001
Workflow	Profiling Strategy - Profiling Target Selection	Profile only non-identified Precursor
Workflow	Profiling Strategy - Profiling Target Selection - Identification Criterion	Qvalue
Workflow	Profiling Strategy - Profiling Target Selection - Threshold	0.001
Workflow	Profiling Strategy - Carry-over exact Peak Boundaries	FALSE
Workflow	Profiling Strategy - Unify Peptide Peaks Strategy	Select corresponding Peak

The Spectronaut output data were used to calculate size-corrected protein intensities with the iBAQ algorithm (parameters: at least 3 unique peptides per protein, precursor Q-value < 0.001, methionine oxidized peptides removed). [7,8] Data analysis and generation of plots was carried out using R[9] (version 4.0.2) depending on the tidyverse [10] [version 1.3.0, Peptides [11] (version 2.4.3), OrgMassSpecR [12] (version 0.5-3), scales [13] (version 1.1.1)]. The localization information of proteins was extracted from reviewed Uniprot entries.

1H nuclear magnetic resonance (1H-NMR) spectroscopic analysis

^{1}H-NMR analysis was performed in 5 mm glass tubes (5 inch in length; Bruker Biospin, Rheinstetten, Germany).[14] Briefly, 400 µL sample volume was buffered to a pH of 7.0 by adding 200 µL of 0.2 M sodium hydrogen phosphate buffer solution, made up with 50% D_2O (Euriso-Top, St-Aubin Cedex, France), which provides an NMR-lock signal. Additionally, the buffer solution contained 1 mM TSP (3-trimethylsilyl-[2,2,3,3-D_4]-1-propionic acid) (Sigma-Aldrich, St. Louis, USA) as an internal standard for subsequent quantification. All NMR spectra were obtained at 600.13 MHz at a temperature of 300 K using a Bruker AVANCE-Neo 600 NMR spectrometer, operated by TOPSPIN 4.0.6 software (both from Bruker Biospin GmbH, Rheinstetten, Germany), using a SampleJet autosampler and a 5 mm QCI cryo probe. A 1D-NOESY pulse sequence was used with pre-saturation on the residual HDO signal during both the relaxation delay and the mixing time. A total of 64 free induction decays (FID scans) were collected using a spectral width of 30 ppm for a one-dimensional spectrum. Spectral referencing was done relative to the TSP signal. 1D spectra were Fourier transformed with a FT size of 32 k and a 1 Hz line-broadening, phased and a polynomial baseline correction was applied over the whole spectral range. Data analysis (identification and quantification) was done by AMIX-Viewer v3.9.15 software (Bruker Biospin GmbH, Rheinstetten, Germany) and Chenomx NMR Suite 8.5 (Chenomx Inc., Edmonton, AB, Canada) using the spectra alignment of pure standard compounds (Sigma-Aldrich, St. Louis, USA). The PROFILER module was used to identify metabolites by fitting the compound signatures from the spectral Chenomx and the Human Metabolome Database HMDB library. Metabolite concentrations were calculated by determining the height of signatures best fitting the experimental signals. Quantification was done using AMIX software 3.9.15 by integration and comparison of metabolite signal peaks to the ERETIC signal which was generated by using external calibration with the ERETIC quantification tool based on PULCON. [15]

Vascular permeability in mice

As described previously,[16] we utilized the Miles edema model [17] to determine vascular leakage in 25-week-old male, wild type C57BL/6 mice. Prior to experiments, mice were anesthetized by intraperitoneal injection of ketamine (120 mg kg-1 BW) and xylazine (16 mg kg-1 BW in saline, 10 mL kg-1 BW). Dorsal skin edema was induced by intradermal injections of 50 µL ChAdOx1 nCoV-19 vaccine (AstraZeneca, LOT ABV3374), 100 µM EDTA (ED2SS, Sigma-Aldrich, Germany), or 0.9% saline (B. Braun, Germany). Then, 0.25% Evans blue dye (10 µL/g in saline, E2129, Sigma-Aldrich) was injected into the retro-orbital plexus. After 30 min, mice were sacrificed by cervical dislocation and skin windows were removed and photographed.

The size of edema was assessed using ImageJ software. To quantitate the extravasated Evans blue tracer, equally sized skin samples were incubated overnight with 500 µL formamide at 55°C [18] and absorbance was measured at 620 nm. Mice were treated according to national guidelines for animal care at the animal facilities of University Medical Center Hamburg-Eppendorf and approved by local authorities (#56/18). All procedures were conducted in accordance with 3Rs regulations.

Digital PCR

Cell-free (cf) DNA from 500-600 µL mouse plasma was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and eluted in 50 µL buffer. Final cfDNA concentrations in the four samples (AZI-III and control) ranged from 21.6 to 34.4 ng/µL. To amplify the adenovirus sequence coding for the codon-optimized spike protein we designed primers for nested PCR using Primer Express version 3.0.1 (Thermo Fisher, Kandel, Germany) **(Supplemental Table S3)**.

Supplemental Table S3: Codon-optimized Spike protein primers for nested PCR.

Primer/Probe	*Sequence*	*Final concentration*
FW1	ccHttYgcHatgcagatggc	100nM
Rev1	gtcatRcaRcaVagcatRat	100nM
FW2	aaYggcacHcaWtggttYgt	100nM
Rev2	ccaRatRtaccaDggccaYtt	100nM
AZ-FP	5´TGGACCTGGGCGATATCAG3´	900nM
AZ-RP	5´TTCAGCCGGTCGATCTCTTT3´	900nM
AZ-P	5´FAM CAATGCCAGCGTCGTGAACATCCA 3´BHQ1	250nM

All primers were obtained from MWG Eurofins (Berlin, Germany). 200 ng genomic DNA, isolated from various tissues 30 min after injection with ChAdOx1 nCov-19, were used as templates. Both first and nested PCRs were performed using Platinum PCR Supermix (Thermo Fisher) for 10 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec, followed by 30 cycles at an annealing temperature of 60°C. A single band of the predicted size (360 bp) was found after nested PCR and Sanger sequencing, using FW2. The obtained sequences showed 100% identity to the published spike sequence. [19] Digital PCR was carried out as duplex PCR and analyzed with the QX100 Droplet Digital PCR System (Bio-Rad,

California). An in-house established reference assay detecting the murine EpoR gene was also included. [20] In order to investigate gDNA amounts >60 ng, we added 5 U of the restriction enzymes *MseI* and *ApaI* (both Thermo Fisher) per individual reaction for mouse gDNA. Data were analyzed with QuantaSoft_v1.7 software (Bio-Rad) including automatic Poisson correction. In order to assess the sensitivity and specificity of dPCR, we used a series of dilutions of the PCR product from the first amplification round of the nested PCR. Sixteen different concentrations were tested. All individual samples with expected copy numbers below 10 were tested in triplicate. [21] To test specificity, gDNA samples from skin tissue at the ChAdOx1 nCov-19 injection site were quantified by duplex dPCR. Of note, five non-vaccinated mice all tested negative for ChAdOx1 nCov-19. Vector copy numbers were normalized per mL plasma. DNA from all other organs was isolated using blackPREP Rodent Tail DNA Kit (Analytik Jena). Vector copy numbers were normalized to 1 µg gDNA based on measured DNA concentrations. A dedicated hydrolysis digital PCR assay (dPCR; TaqMan) was developed that detects ChAdOx1nCoV-19 specific sequences in the spike gene. Digital PCR reactions were performed following our standard protocol. [22]

PF4/heparin ELISA

Binding of immunoglobulin G antibodies from sera of VITT patients to PF4/heparin complexes was measured by a solid phase PF4/heparin ELISA performed in flat-bottomed microwell plates (Cat. No. 478042 Thermo Scientific, CovaLink). PF4/heparin complexes of 0.5 IU/mL unfractionated heparin (UFH; Heparin-Natrium 25000 IE/ 5 mL, Ratiopharm) and 20 µg/mL PF4, in the absence or presence of 20 µM $FeCl_3$, were formed in coating buffer (50 mM NaH_2PO_4, 0.1% NaN_3) at RT for 1 h and incubated for seven days at 4°C. Prior to use, complexes were washed five times with washing buffer (150 mM NaCl, 1% Tween20 pH 7.5). 100 µL/microwell of serum samples (diluted to 1:200 if not indicated otherwise), in sample diluent (0.05 M NaH_2PO_4, 0.15 M NaCl, 7.5% goat normal serum, pH 7.5) were incubated for 60 min, at RT and then washed five times. Horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Europe Ltd, Ely, UK) was added (1:15,000 dilution in sample diluent). Binding of human IgG was detected by adding chromogenic tetramethylbenzidine substrate (100 µL/microwell; Kementec, Taastrup, Denmark). At 60 min post-incubation, the chromogenic substrate reaction was stopped with 1 M H_2SO_4 (100 µL/microwell) for 10 min at RT, and optical absorbance was measured at 450 nm (reference: 620 nm, Tecan, Männedorf, Switzerland) within 10 min. Blank measurements were subtracted from each sample measurement.

Affinity purification of serum IgG by biotin-PF4- and biotin-PF4/heparin-coupled magnetic beads

Coupling of biotinylated PF4 (biotin-PF4; Cat. No. 006/16, Chromatec, Greifswald, Germany) to streptavidin-conjugated paramagnetic microbeads (Dynabeads-SA; Cat. No. 65601, Dynabeads MyOne Streptavidin T1, Invitrogen, USA) was performed according to the manufacturer's instructions. Briefly, 250 µL Dynabeads-SA were washed four times with 1 mL PBS (pH 7.4) and resuspended in 250 µL biotin-PF4 (400 µg/mL PBS). For each sample, 250 µL biotin-PF4 and Dynabeads-SA were co-incubated for 30 min at RT with gentle rotation, and subsequently washed four times with 500 µL washing buffer [PBS pH 7.4, supplemented with 0.1% bovine serum albumin (BSA)]. 200 µL of serum were added and samples were incubated for 90 min at 37°C under gentle rotation. Beads were then washed four times in 500 µL washing buffer. After the last washing step, 400 µL acidic elution buffer (0.1 M glycine, pH 2.7) was added for 1 min. The eluate was subjected immediately to a 100k-centrifugal filter device (Amicon Ultra-2, Merck Millipore, Darmstadt, Germany) and centrifuged for 5 min, at 4000xg. Samples were washed with an additional 400 µL elution buffer, centrifuged again, and the remaining 100-130 µL of supernatant were immediately neutralized with 10 µL Tris-HCl buffer (1 M, pH 9.0). The protein concentration of each sample was measured at 280 nm on a NanoDrop2000 photo spectrometer (ThermoFisher, Waltham, USA) against the respective blank (TRIS-neutralized glycine-buffer). For affinity purification of serum IgG, elution from biotin-PF4/heparin-coupled Dynabeads-SA and PF4/heparin complexes [of 1.0 IU/mL unfractionated heparin (Heparin-Natrium 25000 IE/5 mL; Ratiopharm, Germany) with 40 µg/mL PF4], were formed in 12.5 mL PBS at RT for 1 h. The coupling to washed Dynabeads-Streptavidin (250 µL per sample) was performed consecutively in two steps with 2500 µL of the complex solution (2x 1250 µL) for 30 min each, and with subsequent steps performed as described above.

NETosis assay

EDTA-anticoagulated whole blood from healthy volunteers who did not take anti-platelet medications or non-steroidal anti-inflammatory drugs (NSAIDs) during the previous ten days, was mixed with 5% dextran 500 solution (Serva Electrophoresis GmbH, Heidelberg, Germany), and incubated for 30 minutes at 37°C to sediment red blood cells. The white blood cell-rich supernatant was transferred onto a separating solution (d = 1.077 g/mL; Biocoll, Biochrom AG, Berlin, Germany) and centrifuged at 310 × g for 20 minutes at RT. After the supernatant was discarded, RBCs were lysed for 5 minutes on ice using chilled NH_4Cl lysis

buffer. The cell suspension was then washed twice with PBS without Ca^{2+} and Mg^{2+} (Biochrom AG; 140 × g, 5 min at RT), and neutrophils were resuspended in serum-free RPMI 1640 medium (Cat. No. 21875-034, Gibco, Thermo Fisher Scientific, Germany), and cell concentration was adjusted as required. Neutrophils were stored on ice and used in experiments within 3 h of preparation. Platelets were purified from acid citrate dextrose solution A (ACD-A) anticoagulated whole blood obtained by differential centrifugation from healthy volunteers. Platelet-rich plasma was washed with washing buffer, resuspended in Tyrode's buffer containing 0.35% bovine serum albumin and 0.1% glucose, and platelet concentration was adjusted to 300,000/µL. Washed platelets were stored at 37°C and used in experiments within 3 h of preparation. Neutrophils were resuspended at 1×10^6 /mL in serum-free RPMI media (Cat. No. 21875-034, Gibco, Thermo Fisher Scientific, Germany). *In vitro*, NETosis was studied by incubating neutrophils, alone or with PF4 (10 µg/mL), platelets (1×10^6 /mL), combination of PF4 and platelets, serum from healthy controls (1:50 diluted), or serum from VITT patients (1:50 diluted). In addition, neutrophils were also incubated with IgG from sera of VITT patients (5 µg/mL affinity-purified from biotin-PF4 and biotin-PF4/heparin-coupled magnetic beads), in the presence of platelets, with or without PF4. All incubations were performed on poly-L-lysine coated µ-Slide 18 Well-Flat imaging microchambers (Cat.No:81824, Ibidi, GmbH, Germany) for 60 minutes at 37°C and 5% CO_2. Fixation was performed with 2% PFA for 30 minutes at RT, and slides were gently washed three times in PBS. For fluorescence microscopy, nuclear and extracellular DNA were stained with DRAQ5 (Cat. No. ab108410, Abcam, Germany) used at 5 µM. Platelets were immunofluorescently labelled with FITC mouse anti-human CD61 (Clone RUU-PL7F12, Cat. No. 348093, BD Biosciences, USA) at a final dilution of 0.25 µg/mL. To demarcate the plasma membrane, Lipilight 560 (Membright, Idylle Labs, France) was used at 20 nM. All labeling procedures were performed in PBS for 30 minutes in the dark. Confocal laser microscopy was performed on a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with HCX PL APO λ blue 40.0×/1.25 oil objective. For image acquisition, fluorophores (FITC, Membright 560, and DRAQ5) were excited with Argon-Krypton (488 nm), diode-pumped solid-state (DPSS, 561 nm), and Helium-Neon (HeNe, 633 nm) laser lines that were selected with an acousto-optic tunable filter (AOTF). Fluorescence emission was collected between 505-515 nm for FITC (detector HyD), 566-600 nm for Lipilight 560 (detector PMT), and 640-655 nm for DRAQ45 (detector HyD). Image processing was performed on FiJi (ImageJ version 1.53c). [2,23] Quantification of NETs from fluorescent microscope images was performed using DNA Area and NETosis Analysis (DANA) software for ImageJ and Java. [24] Briefly, fluorescence microscopy images of nuclear and neutrophil extracellular DNA channels were stained with DRAQ5 and imported into DANA_I module in ImageJ, thresholded and segmented automatically to generate regions of interests with gray scale intensities. Percent NETosis was

computed from 12 individual images for each experimental condition acquired from n=3 replicate experiments in the DANA_II Java module.

Quantification of cell-free DNA in serum

Serum samples from VITT patients and healthy donors were diluted 20-fold in phosphate-buffered saline (PBS) containing 0.2% BSA and 4 mM EDTA. Equal amounts of diluted sample and PBS containing 2 µM of the fluorescent DNA-intercalating dye Sytox Green (Invitrogen) were mixed. Fluorescence was measured in a microplate fluorometer (Tecan Spark 10M). DNA concentrations were calculated based on a lambda DNA standard curve (Invitrogen). Statistical analysis was performed using unpaired Student´s *t*-test. Results were considered significant at $P < 0.05$.

Myeloperoxidase (MPO) analysis in serum

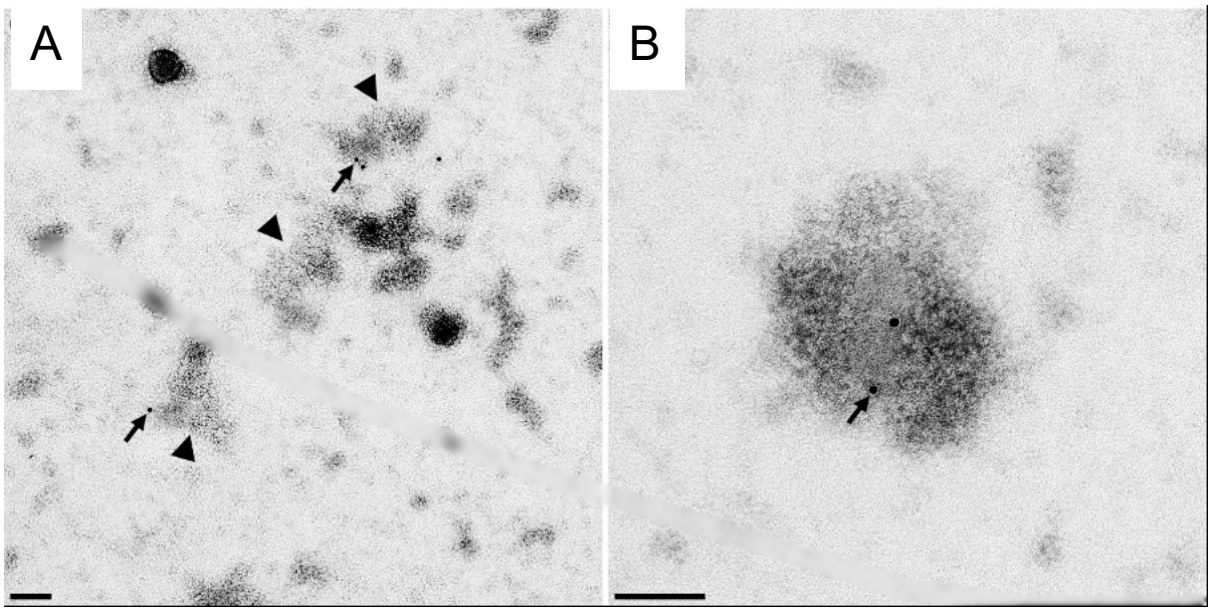
Myeloperoxidase (MPO) concentration was quantified in duplicate from serum samples using a commercial human myeloperoxidase enzyme-linked immunosorbent assay kit (Duoset R&D Systems, DY3174). MPO concentration was determined according to the manufacturer's protocol. In brief, a 96-well microplate was coated with capture antibody overnight, washed, and consecutively blocked for 1 h at RT. The plate was incubated with diluted samples and standards for 2 h at RT. Wells were washed and then incubated for 2 h with detection antibody. Washing was repeated before the streptavidin-HRP working solution was added to wells for 20 min in the dark. Following additional washing steps, HRP-activity was detected using ABTS solution (Life Technologies). The OD was determined using a microplate reader (Tecan Spark 10M) set at 450 nm. Statistical analysis was performed using unpaired Student´s *t*-test. Results were considered significant at $P < 0.05$.

Citrullinated histone H3 measurement in serum

Citrullinated histone H3 (CitH3) concentration was quantified in duplicate from serum samples using a commercial citrullinated histone H3 enzyme-linked immunosorbent assay kit (Cayman chemicals, Clone 11D3, Item No. 501620). The concentration of CitH3 was determined according to the manufacturer´s protocol. In brief, the ready-to-use, pre-coated 96 well plate was incubated with 20-fold diluted samples and standards for 2 h at RT on an orbital shaker. Wells were washed and then incubated with 100 µL of HRP conjugate working solution provided in the kit, for 1 h at RT on an orbital shaker. Washing was repeated as before, and wells were incubated with 100 µL of TMB substrate solution for 30 mins at RT, in the dark, on

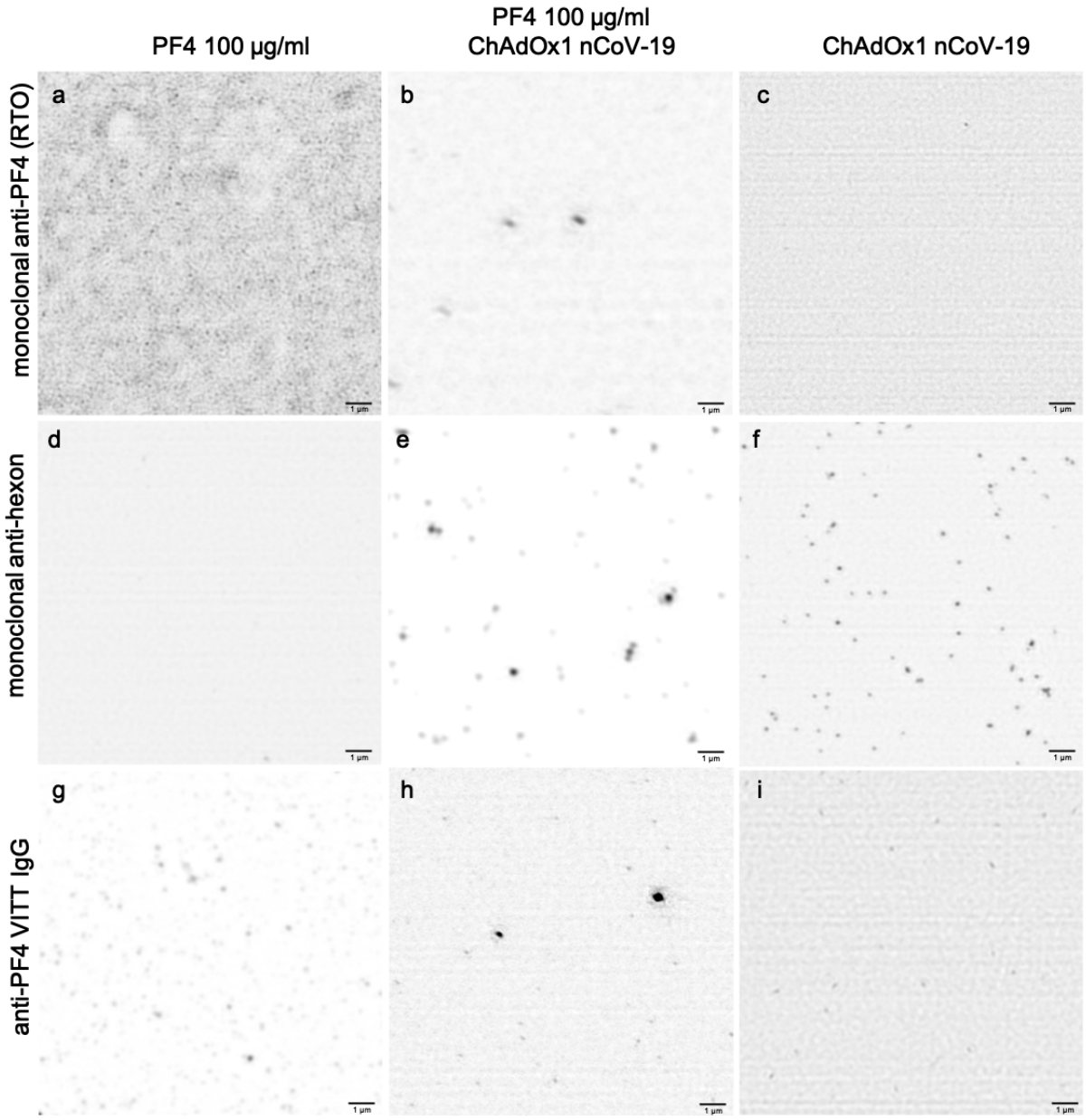
an orbital shaker. 100 µL of HRP stop solution was added to each well and the absorbance was measured using a microplate reader (Tecan Spark 10M) set at 459 nm. Statistical analysis was performed using unpaired Student´s *t*-test. Results were considered significant at $P < 0.05$.

SUPPLEMENTAL FIGURES



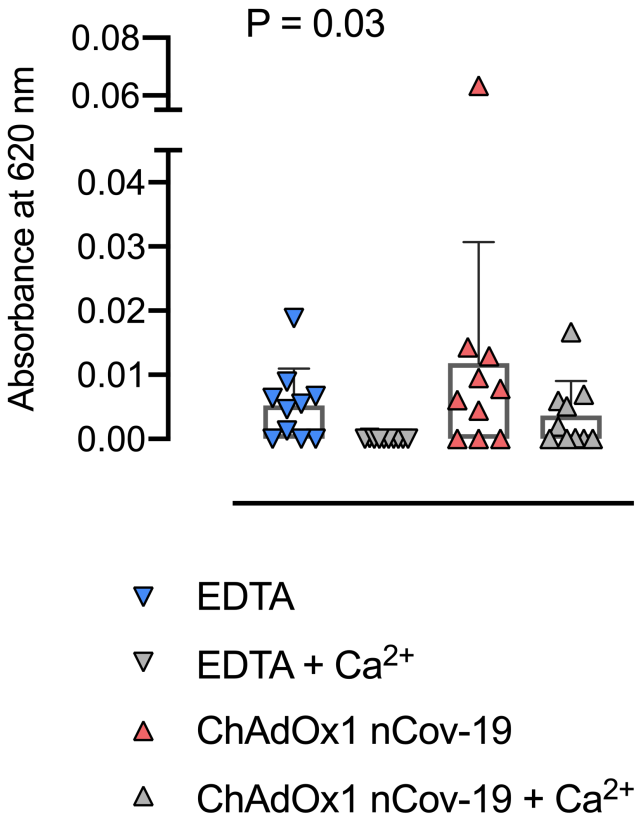
Supplemental Figure S1: Electron Micrograph of PF4 bound to vaccine aggregates.

PF4-biotin was mixed with ChAdOx1 nCov-19 vaccine and probed with Streptavidin-gold. **A**, **B** shows PF4 bound to aggregates in two distinct magnifications. Arrows indicate gold particles and arrowheads show aggregates in the vaccine. Bars represent 100 nm.



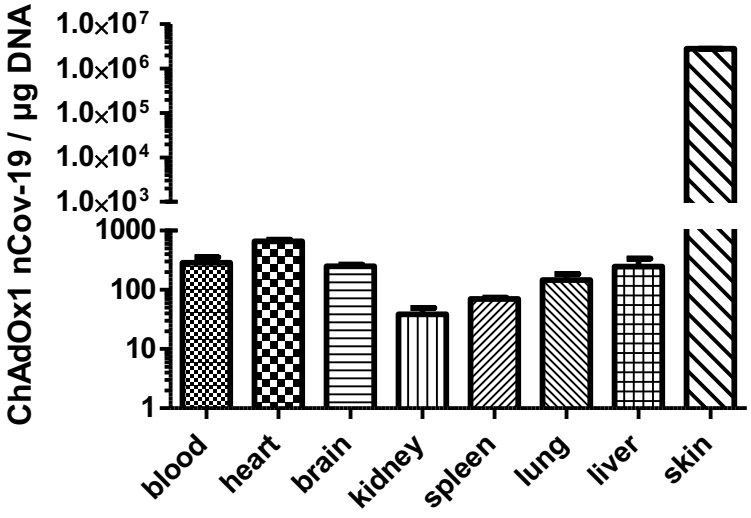
Supplemental Figure S2: Antibody binding controls for 3D-structured illumination microscopy.

Images show super-resolved immunolabelings using anti-PF4 (clone RTO; a, b, c), anti-hexon polypeptide (d, e, f) and human VITT patient-derived purified IgG (g, h, i). Homogenous distribution of immobilized PF4 in the absence of vaccine (a). Formation of dense complexes upon PF4 co-incubation with ChAdOx1 nCoV-19 before immobilization (b). Absence of specific anti-PF4 antibody binding to ChAdOx1 nCoV-19 (c). Absence of anti-hexon polypeptide binding to PF4 (d). Anti-hexon antibody binds to mictures of PF4 and ChAdOx1 nCoV-19 (e) and ChAdOx1 nCoV-19 alone (f). Human VITT patient purified-IgG binds to PF4 (g) and PF4 co-incubated with ChAdOx1 nCoV19 (h) but not ChAdOx1 nCoV-19 alone (i). Scale bars represent 1 µm.



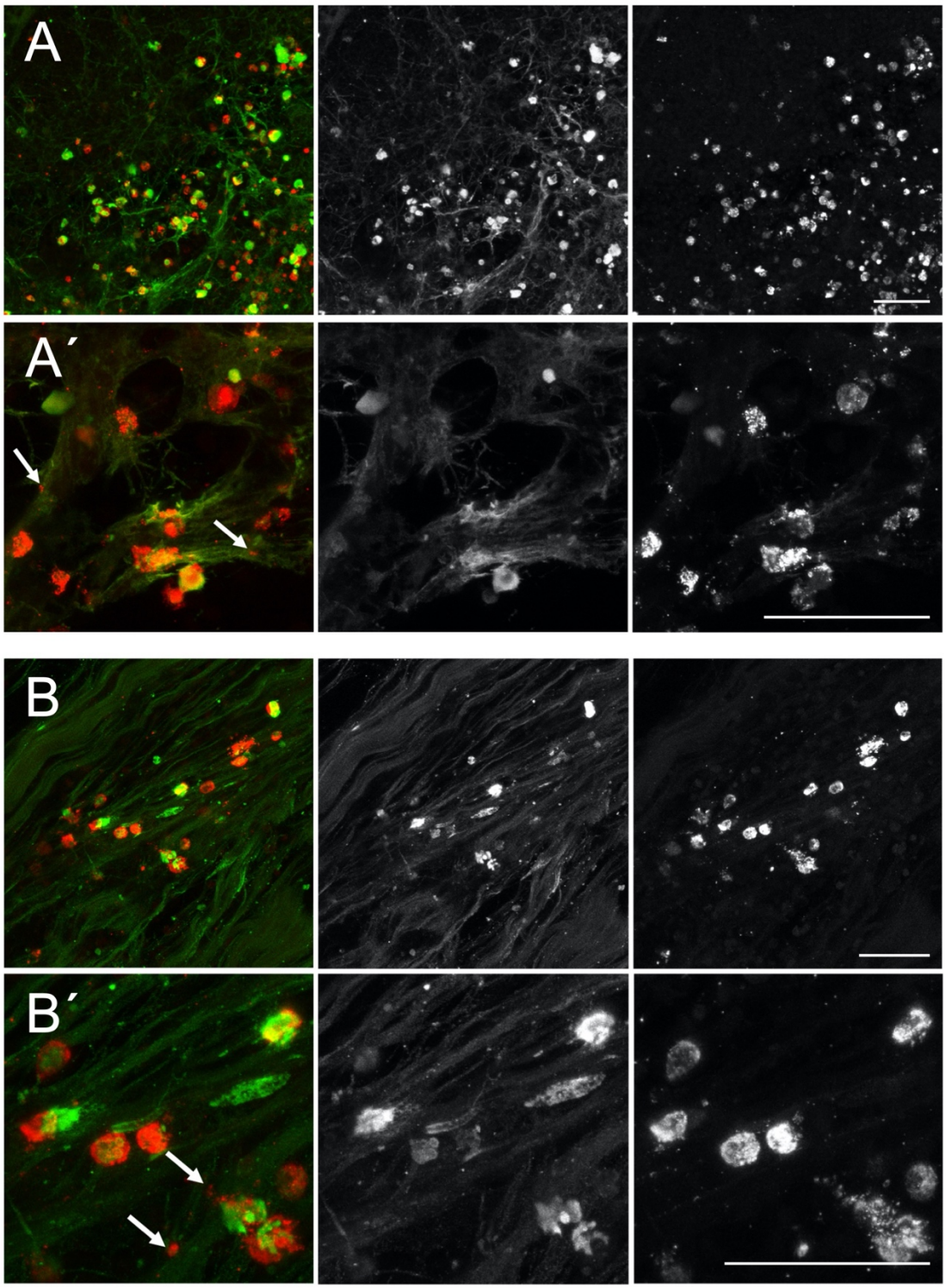
Supplemental Figure S3: Ca^{2+} blunts vaccine-driven vascular permeability.

Skin edema formation in wild-type mice was induced by intradermal injection of 50 µL EDTA (100 µM), ChAdOx1 nCov-19, or saline. To abrogate the biologic activity of EDTA, $CaCl_2$ (100 µM) was added to the intradermal injection solutions to complex EDTA (EDTA + Ca^{2+}) in the vaccine (ChAdOx1 nCov-19 + Ca^{2+}). Evans blue was intravenously infused as a tracer and after 30 min, formed wheels were excised and the extravasated dye was quantified. Columns represent mean ± SD, n = 10 per group. Paired one-way ANOVA followed by Dunn's multiple comparison test was performed for statistical analysis.



Supplementary Figure S4: Leakage of vector DNA 30 min after intradermal injection of 50 µL ChAdOx1 nCov-19.

ChAdOx1 nCov-19 copy numbers in tissues and at the injection site were normalized to µg genomic DNA.



Supplemental Figure S5: NETs in VITT-associated CSVT.

NET formation within a cerebral sinus vein thrombus from a second VITT patient (**A**, **A´**) and VITT-independent CSVT (**B**, **B´**). Materials were obtained by autopsy. Immunofluorescent staining of 100 µm transverse vibratome sections from a cerebral sinus vein thrombus using antibodies against chromatin (green or grey in single channel images) and myeloperoxidase (MPO) (red or grey in single channel images). White arrows indicate NET structures where MPO co-localizes with chromatin-positive DNA strands. Scale bars: 50 µm (**A**, **B**), 10 µm (**A´**, **B´**).

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